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Combination of DNA-directed immobilization and immuno-PCR: very sensitive antigen detection by means of self-assembled DNA–protein conjugates

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ABSTRACT

An assay for very sensitive antigen detection is described which takes advantage of the self-assembly capabilities of semi-synthetic conjugates of DNA and proteins. The general scheme of this assay is similar to a two-sided (sandwich) enzyme-linked immunoassay (ELISA); however, covalent single-stranded DNA–streptavidin (STV) conjugates, capable of hybridizing to complementary surface-bound DNA oligomers, are utilized for the effective immobilzation of either capture antibodies or antigens, rather than the chemi- or physisorption usually applied in ELISA. Immuno-PCR (IPCR) is employed as a method for signal generation, utilizing oligomeric reagents obtained by self-assembly of STV, biotinylated DNA and antibodies. In three different model systems, detecting human IgG, rabbit IgG or carcinoembryonic antigen, this combination allowed one to increase the sensitivity of the analogous ELISA ~1000-fold. For example, <0.1 amol/µl (15 pg/ml) of rabbit IgG was detectable. The immunoassay can be carried out in a single step by tagging the analyte with both reagents for capture and read-out simultaneously, thereby significantly reducing handling time and costs of analysis. Moreover, as the spatial selectivity of target immobilization is determined by the specificity of DNA base pairing, the assay is particularly suited for miniaturized microfluidics and lab-on-a-chip devices.

INTRODUCTION

With the advent of the miniaturization of technical devices, initially driven by the electronics industry, researchers are currently increasingly active in the development of miniaturized microfluidic systems, which enable fast and cost-efficient detection of biomedical and environmentally relevant analytes (1). The miniaturization of ligand binding assays not only reduces costs by decreasing reagent consumption but also leads to enhanced sensitivity as compared with macroscopic techniques. While the microfluidic analysis of nucleic acids, conducted by microchip PCR and/or microarray-based hybridization (2–5), has made large steps towards routine application, the analysis of proteins in microstructured devices is hampered by the instability of most proteins. Although protein microarrays have been prepared for high-throughput antibody screening (6), analysis of antibody–antigen interactions (7) and identification of the protein targets of small molecules (8), the stepwise, robotic immobilization of multiple proteins at chemically activated surfaces is often obstructed by the instability of most proteins which usually reveal a significant tendency for denaturation, and thus, loss of functionality. This problem is particularly severe in the fabrication of protein functionalized microstructures, since a microfluidic element usually needs additional fabrication steps, i.e. the bonding of a lid, subsequent to the immobilization of the bioactive proteins. In addition to solving the problem of mild and spatially selective immobilization of protein reagents for immunoassays, a maximum sensitivity as well as a large dynamic range of quantification are required to account for small concentrations of many antigens.

Here we report on a fast and highly sensitive immunoassay based on semi-synthetic conjugate reagents comprised of DNA and proteins. As indicated in Figure 1, the general scheme of this assay is similar to a two-sided (sandwich) enzyme-linked immunoassay (ELISA). However, while in ELISA the capture antibodies or the analyte are bound to the solid-phase by chemi- or physisorption, we here employ the method of DNA-directed immobilization (DDI) of proteins (9) using covalent conjugates synthesized from single-stranded DNA (ssDNA) and streptavidin (STV) as molecular adapters (10,11). DDI provides a chemically mild process for the site-selective adsorption of delicate proteins to a solid support, using DNA-functionalized substrates as an immobilization matrix. Because the lateral surface structuring can now be carried out at the level of stable nucleic acid oligomers, the DNA-functionalized substrate can be further fabricated and stored almost indefinitely, functionalized with proteins of interest via DDI immediately prior to its use in, for example, immuno analytics. Moreover, subsequent to the immunoassay,

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Dedicated to Professor Manfred T. Reetz on the occasion of his 60th birthday

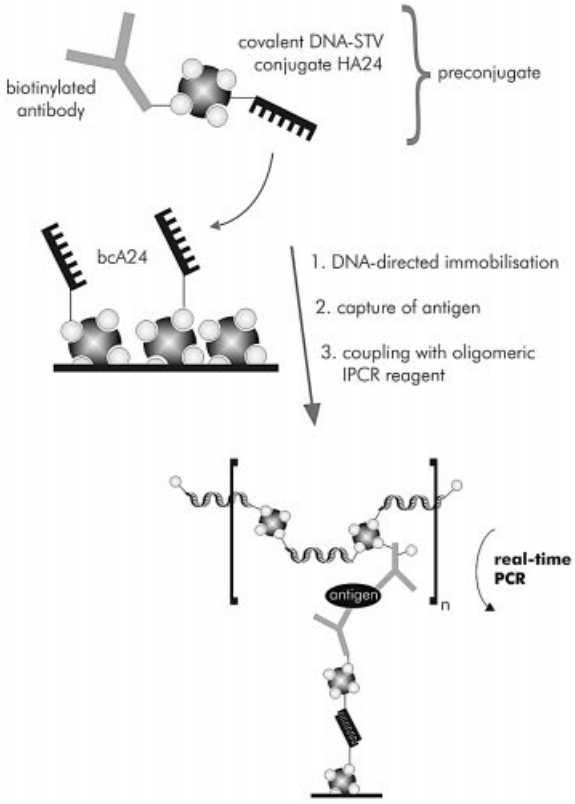

Figure 1. Schematic representation of the immunoassay based on DDI and IPCR. A covalent DNA–STV conjugate, HA24, is coupled with a biotinylated antibody by mixing the two compounds, thereby generating a preconjugate. Biotinylated antisense capture-oligonucleotide bcA24 was immobilized on STV-coated microplates and the remaining free biotin-binding sites of the surface-bound STV are blocked with D-biotin, represented by shaded spheres. The preconjugates are then allowed to bind to their complement by specific DNA hybridization. The immobilized antibody is used for capturing the antigen and the amount of target analyte is determined by IPCR, using oligomeric conjugates comprised of STV, bis-biotinylated dsDNA and biotinylated antibodies. The read-out of immunoassay is carried out using real-time PCR.

the substrate can be regenerated by alkaline denaturation of the double-helical DNA linkers. As an additional advantage of DDI in immunoassay applications, we demonstrate here that the binding of the target antigen by antibodies can be carried out in homogeneous solution, instead of in a less efficient heterogeneous solid-phase immunosorption. Subsequently, the immuno-complexes formed are captured at the DNA-functionalized substrate by nucleic acid hybridization.

To account for the small concentrations of many antigens present in clinical or environmental samples and to realize a large dynamic range of quantification, we employed immuno-PCR (IPCR) as a high sensitivity read-out in the DDI-based immunoassay. IPCR, first described by Sano *et al.* (12), allows the ultrasensitive analysis of proteins and other antigens by combining the well established ELISA methodology with the signal amplification power of the PCR. As a consequence, IPCR not only leads to an ~1000–10 000-fold gain in sensitivity, as compared with analogous ELISA detection, but IPCR also reveals a very broad linear dynamic range of up to six orders of magnitude (13–16). The key reagents of IPCR, conjugates of antibodies and a DNA marker fragment, can be synthesized by covalent cross-linking (17) or self-assembly, for example, using the high-affinity interaction of STV and biotin (13,18,19). With respect to the latter approach, we had previously reported on the development of self-assembled conjugates, prepared from STV, bis-biotinylated double-stranded (dsDNA) fragments and biotinylated antibodies, which showed an enhanced performance as reagents in IPCR (13). The applicability of such conjugates in routine immunodiagnostics has recently been demonstrated for pharmacokinetic studies (20) and the high-throughput protein detection employing real-time PCR quantification of the IPCR amplicons (21).

The combination of DDI and real-time IPCR described here allows one to detect <0.1 amol/µl (15 pg/ml) of antigen, corresponding to an ~1000-fold enhancement of the sensitivity of the analogous ELISA detection. Due to the high sensitivity detection capabilities and the robustness of the real-time IPCR read-out, which is combined with the site-selective protein immobilization provided by DDI, we anticipate that the DDI–IPCR assay described here, not only is applicable as a rapid and versatile test in the common microplate format, but is also suited for the novel microfluidics and lab-on-a-chip platform, which is currently advancing to the cutting edge of bioanalytics and engineering.

MATERIALS AND METHODS

Preparation of DNA–protein conjugates

Synthesis and purification of covalent DNA–STV conjugate HA24 was carried out from the corresponding thiolated oligonucleotide, tA24 (5′-thiol-TCC TGT GTG AAA TTG TTA TCC GCT-3′), as previously described (11). In brief, STV (10 nmol) was derivatized with maleimido-groups using a heterobispecific cross-linker (sulfo-SMPB; Pierce), reacted with tA24 (10 nmol) and subsequently purified by anion-exchange chromatography. The one-to-one molar ratio of oligonucleotide and protein moiety of HA24 was verified by gel-electrophoretic and photometric analysis, and its concentration was determined by absorbance measurements (11).

Preconjugates of HA24 and biotinylated antibodies were prepared by mixing 0.01 mM stock solutions of HA24 and equimolar amounts (0.01 mM stock solution) of biotinylated goat anti-rabbit IgG (Sigma), biotinylated goat anti-human IgG (Biotrend) or biotinylated rabbit anti-CEA (Dako) [biotinylated with NHS-Biotin (Pierce) according to the manufacturer's instructions] in buffer A (10 mM Tris buffer, pH 7.5, containing 5 mM EDTA). After incubation for 20 min at room temperature, the mixtures were diluted to final concentrations, typically in the range of 50–1 nM, with buffer B [20 mM Tris–Cl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.01% (w/v) Tween-20] containing 0.1 mg/ml reagent grade DNA (Roche), 800 µM D-biotin (Sigma) and 0.5% milk powder (Oxoid) and the dilutions were incubated for an additional 10 min.

Supramolecular reagents for IPCR were prepared from bis-biotinylated DNA, STV and biotinylated antibodies, as previously described (13). Briefly, recombinant STV (Roche) and a 256 bp bis-biotinylated dsDNA fragment which contains a biotin group at both 5′ ends were typically

prepared by adding 4 µl of STV (20 µM in buffer A) to ~60 µl of buffer A, and subsequently, 4 µl of the dsDNA (10 µM in buffer A). Following incubation for 20 min at room temperature, two molar equivalents of the biotinylated antibody were added to the oligomeric DNA–STV conjugate. The mixture was incubated for 30 min at room temperature and the DNA–protein aggregate was purified by fast-protein liquid chromatograhy using a Superdex 200 gel-filtration column (Pharmacia).

DNA-directed immobilization

Thirty microliters of a 240 nM solution in buffer B of the biotinylated capture oligomer (5′-biotin-AGC GGA TAA CAA TTT CAC ACA GGA-3′, bcA24), complementary to HA24, were incubated in STV-coated wells of TopYield microtiter modules (Nunc, Roskilde, Denmark), prepared as previously described (14). Similarly, a non-complementary capture oligomer (5′-biotin-ATG TGA CCT GTA TTG TTG GAT GTG AG-3′) was immobilized as a control. The oligomers were allowed to bind for 30 min. After washing with buffer B containing 800 µM D-biotin, 30 µl of the HA24-IgG preconjugate solution, described above, was allowed to hybridize for 30 min at room temperature. The plate was washed four times with buffer B and 30 µl of a serial dilution of antigen [IgG from human or rabbit (Sigma) or carcino-embryonic antigen (CEA; Dako)], typically ranging from 10 000 amol/µl (1.5 µg/ml) to 0.01 amol/µl (1.5 pg/ml) in buffer A containing 0.1 mg/ml reagent grade DNA (Roche) and 0.5% milk powder (Oxoid) for IgG and ranging from 1000 amol/µl (0.2 µg/ml) to 0.01 amol/µl (2 pg/ml) in standardized human serum (Biseko) for CEA, were added and incubated for 45 min. The plate was washed four times with buffer B and immediately used for ELISA and real-time IPCR detection, respectively, carried out directly in the microplate. Nunc Top Yield modules fit into the ABI Prism™ 7000 instrument.

ELISA

In the case of ELISA, signals were generated using fluorogenic AttoPhos, as described before (20). In brief, subsequent to washing of the plate, 30 µl of a conjugate produced from biotinylated antibody and covalent STV–alkaline phosphatase conjugate (Roche) was incubated for 30 min in the wells containing the serial dilution of the antigens. Signals were generated using fluorogenic AttoPhos (Roche) and fluorescence detection in a microtiter plate reader (Perkin-Elmer).

Real-time IPCR

IPCR was carried out as described before (13,20). In brief, 30 µl of the antibody-containing DNA–protein IPCR reagent described above [~500 pM, in buffer B, containing 0.1 mg/ml reagent grade DNA (Roche), 800 µM D-biotin (Sigma) and 0.5% milk powder (Oxoid)] were incubated for 30 min in the wells previously treated with serial dilutions of the antigens described above. Subsequently, the wells were washed seven times with buffer B and twice in buffer C (20 mM Tris–Cl buffer, pH 7.5, 150 mM NaCl).

The read-out of the IPCR amplicon products was achieved by either PCR–ELISA, carried out similar to that described previously (14), or by real-time PCR using the TaqMan principle (21). The latter was carried out using the ABI Prism™ 7000 sequence detection system (Applied Biosystems).

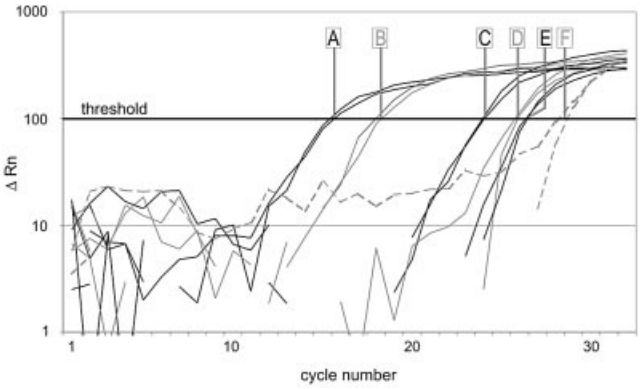

Figure 2. Real-time PCR amplification curves of the DDI–IPCR assay for the detection of rabbit IgG (rIgG). Representative samples of different concentrations of the rIgG antigen, analyzed in duplicate by the DDI–IPCR assay, are shown. The TaqMan software calculates the threshold cycle at which the fluorescent reporter signal exceeds the signal of the threshold. This threshold cycle is set at 100 ΔRn. Data points A–D represent different rIgG concentrations of 10 nM (A), 100 pM (B), 1 pM (C) and 10 fM (D), respectively. E indicates the NC of the IPCR (no rIgG antigen present during immuno-capture) and F the NC of the PCR (amplification of pure PCR reagent without the presence of any template). Note the high reproducibility of the real-time curves with a standard deviation of <8% (mean 1.6%) for duplicate measurements. Four-fold independent repetition of this assay, carried out under identical conditions, also revealed an inter-experimental error of <8% (mean 4.5%).

As shown in Figure 2, this instrument monitors the increase of the normalized reporter signal (Rn) for each cycle. A baseline correction was applied, using typically the first 12 cycles as background signal. The software calculates the threshold cycle (C_t), which represents the first PCR cycle at which the reporter signal exceeds the signal of the baseline determined by the user. The threshold level was determined to be above the background signals for empty wells and was set in the linear signal increasing phase according to the data obtained for each experiment. The threshold value was set at 100 ΔRn in the experiments described here. The normalization of the C_t values was achieved as follows. As C_t values are inversely proportional to the amount of DNA template, and thus, antigen concentration, the C_t values were subtracted from the maximum number of cycles in the PCR, that is 40, to generate ΔC_t values. These values are increasing with increasing amount of target DNA and antigen, respectively, thus facilitating the comparison of data generated from PCR–ELISA and real-time PCR. Since each IPCR sample was analyzed in duplicate, the mean values and standard deviation of ΔC_t values were calculated for each double determination. All values for the ELISA and IPCR measurements were finally normalized on the respective IPCR negative controls (NC), containing no antigen (Fig. 2). Thus, NC data points are fixed at a value of 1.0.

RESULTS AND DISCUSSION

To facilitate the DDI, the covalent conjugate HA24 was synthesized from a 24mer ssDNA oligonucleotide and

recombinant STV. Synthesis and purification of HA24 was carried out from the corresponding thiolated oligonucleotide, tA24 (for sequences, see Materials and Methods) using a heterobispecific cross-linker, as previously described (11). The covalent DNA–STV conjugate can be used as a versatile molecular adapter, allowing for convenient tagging of biotinylated proteins with a single-stranded oligonucleotide. To employ the DDI method for the functionalization of a microplate with capture antibodies, preconjugates are produced from HA24 and one molar equivalent of the biotinylated antibody leading to the formation of a preconjugate, capable of hybridizing to surface-bound capture oligonucleotides (Fig. 1). STV-coated microplates were used as a solid support for capturing, and to this end, were functionalized with biotinylated oligonucleotides, complementary to the DNA sequence in HA24. In a first set of experiments, the functionalization of the capture plate was investigated using biotinylated antibodies from goat directed against human IgG (GAH). To estimate the immobilization efficiency of the DDI, similar amounts of the biotinylated GAH were immobilized either directly in wells of microplates by physisorption, by means of the STV–biotin interaction in wells of the STV-coated microplate or by DDI, using the oligonucleotide-functionalized STV microplates (Fig. 3).

Subsequent to immobilization of the GAH, serial dilutions of human IgG as a target antigen were incubated in the wells containing fixed amounts of the GAH capture antibody. Signal detection was first carried out by regular ELISA. As shown in Figure 3A, the capture plates produced by DDI led to the best detection limit of <30 amol/µl hIgG, corresponding to 4.5 ng/ml. Immobilization by physisorption and STV–biotin interaction led to slightly worse results, with a detection limit of ~100 amol/µl hIgG (15 ng/ml). This result is in agreement with prior studies of DDI (10), in which the high efficiency of protein immobilization had been attributed to: (i) the reversibility of DNA hybridization, enabling a denser packing on formation of the protein layer; (ii) the lean structure of the rigid double-helical DNA spacer between the surface and the protein which may also contribute to a larger effective surface area; and (iii) to a higher biological activity, which may result from the larger distance between surface and antibody, enabling a more homogeneous type of reaction during the formation of the immuno-complex.

In a second set of experiments, the capture plates produced by the methods described above were used in an IPCR assay (Fig. 3B), employing pre-synthesized oligomeric conjugates comprised of biotinylated GAH, recombinant STV and bis-biotinylated dsDNA marker fragments (13). Detection of PCR amplicons was achieved using TaqMan real-time PCR quantification (21). Similar to the ELISA case, the performance of capture plates prepared by DDI exceeded those prepared by physisorption or STV–biotin interaction. In particular, DDI allowed a detection limit of ~0.3 amol/µl hIgG, corresponding to 45 pg/ml, while physisorption and STV–biotin interaction were slightly worse (~3 amol/µl, 450 pg/ml). Owing to the high sensitivity of IPCR, the detection limit of all three IPCR assays was found to be ~100-fold better than in the case of the ELISA analysis.

To further demonstrate the applicability of DDI-based immunoassays, the tumor marker human CEA (22) was chosen as a target. In a model study, serial dilution samples

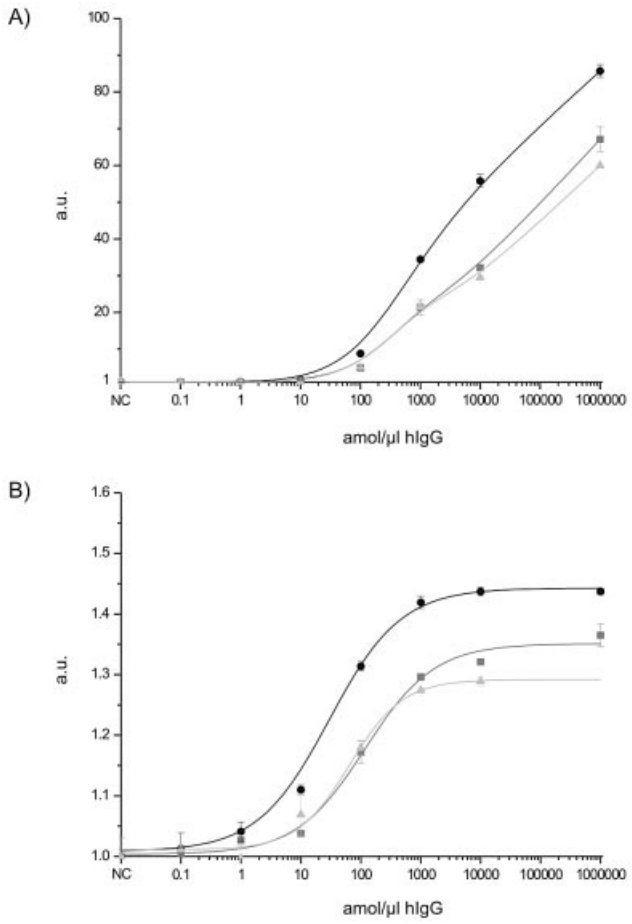

Figure 3. DDI of capture antibodies from goat directed against human IgG (GAH). Immobilization efficiencies of three alternative techniques are compared: biotinylated GAH was immobilized by either direct physisorption (triangles), biotin–STV interaction (rectangles) or DDI (circles). Serial dilutions of the target antigen (human IgG, hIgG) were incubated in the wells containing the capture antibody. Signal detection was carried out by either regular ELISA (**A**), using an anti-hIgG–STV–alkaline phosphatase conjugate and fluorescence detection or IPCR (**B**) using an anti-human IgG-STV-DNA conjugate and real-time TaqMan detection. The standard deviation of duplicate measurements was <5%, e.g. 1.04 ± 0.016 for detection of 1 amol/µl hIgG (B, closed circles). Note the ~100-fold increase in sensitivity associated with the use of IPCR instead of ELISA signal detection.

were prepared by spiking normalized human plasma (BISEKO) with purified CEA. Commercially available polyclonal anti-CEA antibody (ACA) from rabbit was biotinylated, coupled to the HA24 conjugate, and the resulting preconjugate was used for preparing ACA-functionalized microplates by DDI. Serial dilutions of the CEA in blood serum were incubated in the wells containing the ACA, and signal detection was carried out either by regular ELISA using an ACA–STV–alkaline phosphatase conjugate and fluorogenic AttoPhos or by IPCR, using an oligomeric ACA–STV–bis-biotinylated dsDNA conjugate (13) and real-time TaqMan PCR. As shown in Figure 4, the limit of detection for CEA was ~50 amol/µl (10 ng/ml) CEA for DDI–ELISA, while with the DDI–IPCR method, an ~1000-fold increase in sensitivity was obtained, allowing the detection of ~0.05 amol/µl (10 pg/ml) of CEA. In comparison, a regular ELISA assay, based on

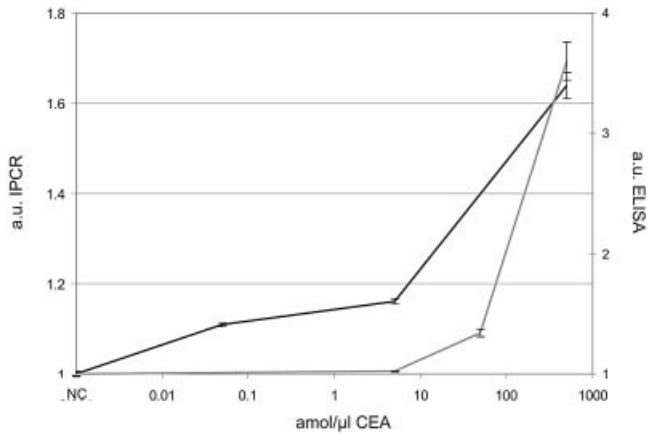

Figure 4. Application of the DDI–IPCR combined assay for the detection of CEA. The signals obtained for serial dilutions of CEA in human blood serum are compared for two different assays, i.e. the DDI–ELISA (light gray) and DDI–IPCR (black). The standard deviation in IPCR is typically <5%, e.g. 1.16 ± 0.006 for the detection of 5 amol/µl CEA (black). Note that IPCR detection leads to an ~1000-fold enhancement in the limit of detection.

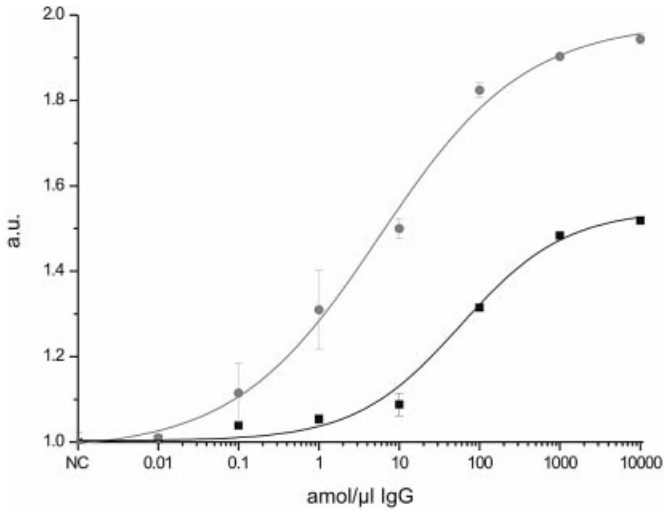

Figure 5. Detection of IgG from human (hIgG, black rectangles) and rabbit (rIgG, light-gray circles) by means of the DDI–IPCR assay. The standard deviation of duplicate measurements was <8%, e.g. 1.31 ± 0.093 for detection of 1 amol/µl rIgG (gray circles). Note the increased sensitivity for the detection of rIgG, as compared with hIgG.

physisorbed ACA, only allowed the detection of ~500 amol/µl (100 ng/ml) of CEA (data not shown). These results clearly confirm the robustness and sensitivity of the DDI-based immunoassays, making them applicable to the detection of antigens in highly complex matrices, such as blood serum.

As an additional test of applicability, IgG from rabbit (rIgG) was chosen as a target antigen. For this, biotinylated goat anti-rabbit IgG (GAR) antibody was coupled with HA24 and the resulting preconjugate was used in the DDI-based functionalization of a microplate. Serial dilutions of the rIgG antigen in buffer were allowed to bind and the target adsorbed to the surface was quantified by real-time IPCR, employing a preformed conjugate comprised of biotinylated GAR, STV and bis-biotinylated dsDNA (13). In a parallel assay, the detection of IgG from humans (hIgG) was conducted for comparison. As indicated in Figure 5, the detection of rIgG led to much better results, as compared with those obtained for hIgG. As little as 0.03 amol/µl (4.5 pg/ml) of rIgG was detectable, while the analysis of hIgG only allowed the detection of 1 amol/µl (150 pg/ml) of antigen. The alterations in sensitivity reflect individual differences in the kinetic and thermodynamic binding properties of the two antibodies, GAR and GAH, employed in the immunoassays.

A general advantage of DDI-based immunoassays relies on the high-specificity immobilization mediated by the base pairing of complementary nucleic acids. This even allows for simultaneously immobilizing many different DNA-tagged complexes site-specifically on a DNA microarray (9). Thus, DDI-based immunoassays, in principle, allow that the binding of the antigen target by DNA-tagged antibodies is carried out in homogeneous solution, and subsequently, the immunocomplexes formed are captured at the DNA-functionalized substrate by nucleic acid hybridization. This approach not only would reduce the number of incubation steps, but it also might lead to increased sensitivity, since the antibody–antigen interaction occurs faster in homogeneous solution than it does at the solid/liquid interphase in regular immunoassays.

To investigate these types of in-solution capture assays, we carried out a series of experiments using the rIgG/GAR system (Fig. 6). Initially, a two-step assay was investigated. To this end, serial dilutions of rIgG in buffer were mixed with fixed amounts of the HA24–GAR preconjugate. To study how capture reagent concentration influences the sensitivity of the immunoassay, three different concentrations of the HA24–GAR were used, ranging from 2, 10 to 50 nM (Fig. 6A). The mixtures of the HA24–GAR with the rIgG were immediately applied to the wells of a microplate containing capture oligonucleotides, thereby allowing for the DNA-directed adsorption of the immuno-complexes formed in solution. Subsequent to incubation for 30 min, the plate was washed and the rIgG immobilized was quantified by real-time IPCR using a 500 pM solution of a conjugate, comprised of biotinylated GAR, STV and bis-biotinylated dsDNA (13). As expected, both the signal intensities and sensitivity of the assay clearly depended on the concentration of capture reagent (Fig. 6A). A best sensitivity of ~0.1 amol/µl (15 pg/ml) was obtained for 10 nM of capture conjugate, while both an increase to 50 nM and a decrease to 2 nM of capture conjugate led to a reduced sensitivity of 300 amol/µl (45 ng/ml) and 2 amol/µl (300 pg/ml), respectively. However, the signal-to-noise ratio was not as good as that obtained in the analogous three-step assay (Fig. 5). To explain the HA24–GAR concentration-dependent assay sensitivity in Figure 6A, one needs to consider the relative stoichiometric amount of HA24–GAR conjugate complexes and rIgG molecules. At high ratios of HA24–GAR:rIgG, binding sites of rIgG are likely to be blocked, and consequently, binding of the IPCR detection reagent is sterically hindered. Thus, lower IPCR signals result. At low ratios of HA24–GAR:IgG, incomplete oligonucleotide tagging of the antigen occurs, and thus, the sensitivity is decreased.

To study the feasibility of a one-step immunoassay, serial dilutions of the rIgG antigen were mixed with a fixed amount of HA24–GAR capture reagent, either 4 or 10 nM,

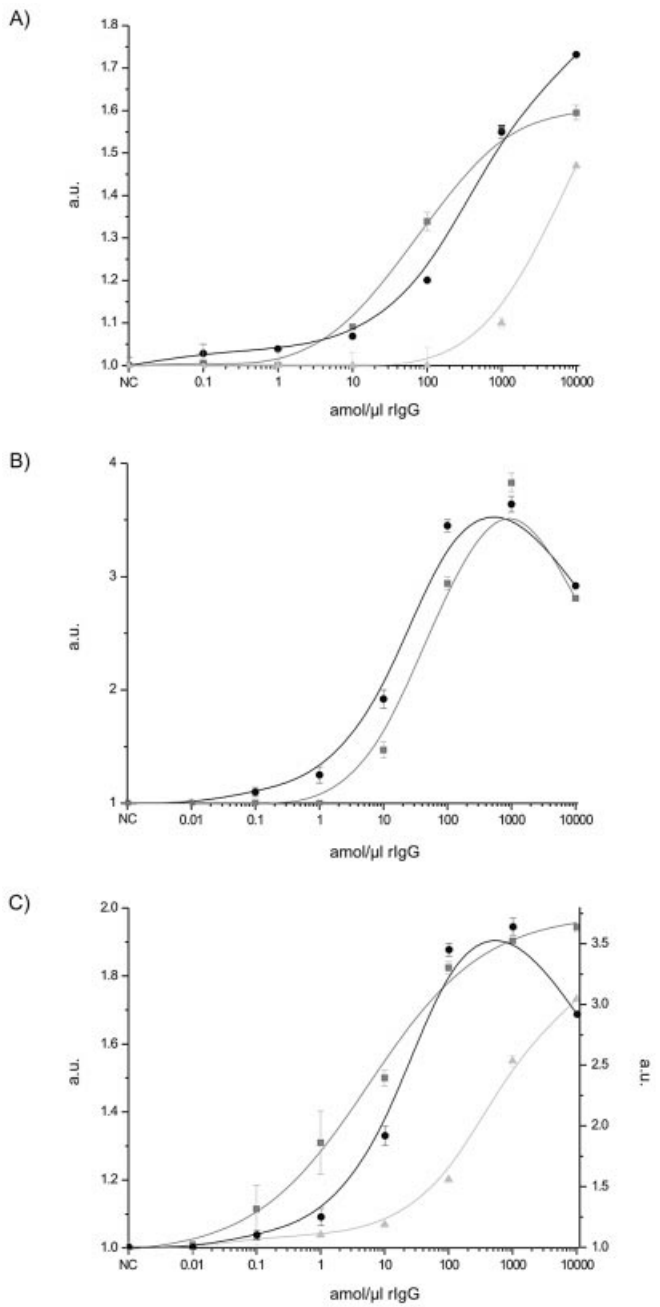

Figure 6. Performance of DDI-based in-solution capture assays for the detection of rIgG. (**A**) Two-step immunoassay: serial dilutions of the rIgG analyte were mixed with varying amounts of capture reagent HA24-GAR, ranging from 2 (rectangles), 10 (circles) to 50 nM (triangles). The immuno-complexes formed in solution were immobilized in DNA-functionalized microplates, IPCR detection conjugate was bound, and signal detection was achieved by real-time PCR. (**B**) One-step immunoassay, carried out by mixing serial dilutions of rIgG with a fixed amount of capture reagent (circles, 4 nM; rectangles, 10 nM) and IPCR detection conjugate (25 pM). (**C**) Comparison of the three different DDI–IPCR assays investigated, requiring either three steps (rectangles), two steps (triangles) or one step (circles) of reagent incubation. The two- and three-step assays were obtained using 10 nM of capture reagent, while 4 nM was used in the one-step assay. Note that the standard deviation is typically <8%, e.g. 1.25 ± 0.07 for the detection of 1 amol/µl rIgG in the one-step assay (C, circles).

respectively, and 25 pM of the IPCR detection conjugate, comprised of the biotinylated GAR, recombinant STV and bis-biotinylated dsDNA. The mixtures were immediately applied to oligonucleotide-coated microplate wells to allow for the DNA-directed adsorption of the immuno-complexes formed in solution. Subsequently, quantification of signals was carried out by PCR and PCR–ELISA. As indicated in Figure 6B, signal intensities and the assay's overall sensitivity strongly depended on the amount of capure reagent. The lower concentration of capture reagent, 4 nM HA24–GAR, led to an increased detection limit of 0.1 amol/µl (15 pg/ml) of rIgG, as compared with 2 amol (300 pg/ml), respectively, obtained in the case of 10 nM HA24–GAR. In both cases, an unusually shaped dose–response curve was evident, indicating a significant decrease in signal intensity when high amounts of rIgG antigen were present in the sample (see curves in Fig. 6B). This shape was highly reproducible, and most likely, is caused by limited amounts of the detection conjugate. This leads to an incomplete labeling of antigen molecules, and thus, decreased signal intensities. Unfortunately, this effect cannot be compensated for by increasing the detection conjugate concentration. Since IPCR is highly sensitive to the concentration of the detection conjugate, i.e. the amount of marker DNA incubated in the reaction vessel (12,13), an increase in detection conjugate concentration was inevitably associated with high background signals, and thus, a dramatic decrease in sensitivity (data not shown).

Figure 6C allows one to directly compare the conventional three-step assay, exclusively based on successive reagent incubation steps, with the corresponding two- and one-step assays described above. It is clearly evident that the decrease in incubation steps is associated with a decrease in the overall sensitivity of the immunoassay. As discussed above, signal intensities depend on the relative amounts of target, capture and detection reagents. Consequently, best results are obtained with the conventional protocol, taking advantage of the repeated reagent incubation and washing steps. The in-solution capture assays, however, not only allow for a significant reduction in assay time but also still offer a significant increase in sensitivity, as compared with conventional ELISA detection. Additional enhancement in sensitivity might well be achieved by systematic optimization of assay conditions, in particular, the concentration of reagents.

CONCLUSIONS

Here, we reported on the development of an immunoassay for the high sensitivity detection of antigens. The assay extensively utilizes the self-assembly capabilities of semi-synthetic DNA–protein conjugates. In particular, covalent ssDNA–STV conjugates are employed as molecular adapters for the effective DNA-directed immobilization of capture antibodies at solid supports containing complementary oligonucleotides. The capture antibodies are used for the selective capture of the analyte, similar to conventional sandwich ELISA. Subsequently, IPCR is employed as a high sensitivity detection method, taking advantage of conjugates produced by self-assembly of STV, biotinylated dsDNA and antibodies directed against the analyte. In three different model systems, the DDI–IPCR assay allowed us to detect low amounts of antigen, typically 100–1000-fold less than that detectable by

conventional sandwich ELISA. While this increase in sensitivity is mainly due to the IPCR step, distinct positive effects of the DDI have been demonstrated regarding the immobilization efficiency of the capture antibody. The DDI–IPCR assay reveals a remarkable robustness and reproducibility with respect to intra- and inter-assay deviations. In addition, we have shown that the immunoassay can even be carried out in a single step by simultaneously tagging the analyte with both capture and detection reagents, thereby significantly reducing handling time. Moreover, as the spatial selectivity of target immobilization is controlled by DNA hybridization, the assay is well suited for the parallelized detection of several antigens, using, for instance, microfluidic channels and lab-on-a-chip devices, currently under development (e.g. for multiplex PCR assays).

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